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June 9, 2011

Dr. Lori White
NTP Office of Liaison, Policy and Review
National Institute of Environmental Health Sciences
PO Box 12233, MD K2-03
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Dear Dr. White:

The following comments are submitted on behalf of the more than two million members and supporters of the Physicians Committee for Responsible Medicine (PCRM) and People for the Ethical Treatment of Animals (PETA) in response to the nominations of *in vitro* botulinum and *in vitro* pyrogen assays to ICCVAM for validation. (April 26, 2011; Federal Register 76[80]:23323). Our organizations are committed to replacing and reducing animal use with the best available *in vitro* and computational science.

MAT Pyrogenicity Assay

We support an expanded domain of applicability for the nominated MAT, but we have suggestions for how to proceed with validation that differ from those in the nomination documented submitted. A summary of the major recommendations are listed below, followed by more detailed comments.

Major Recommendations

1. Coordinate and collect data from smaller, product-specific validation studies from individual companies that still use the RPT rather than require that a large validation study be conducted.
2. Expand efforts to gather information on pyrogenicity testing from manufacturers of parenterals, biologics, and devices as well as the FDA
3. Perform a current literature review on the immune response to pyrogens.

Background

BioTest has nominated its Monocyte Activation Test (MAT), an IL-1 β enzyme-linked immunosorbent assay (ELISA) using cryopreserved blood, for broader validation. This assay was one of the five *in vitro* pyrogenicity assays approved by ICCVAM in 2008 for

the detection of pyrogenic contamination. These five methods were approved for the detection of Gram-negative endotoxin in human parenteral drugs, subject to product specific validation. BioTest has proposed a validation study to expand the domain of applicability to include non-endotoxin pyrogens as well as products other than parenterals, including devices and biologics, with the intent of fully replacing the rabbit pyrogen test (RPT). Although there were abundant data on the ability of the five methods to detect both endotoxin and non-endotoxin pyrogen in a variety of product classes, ICCVAM found the data inadequate for validation purposes, and limited the validation to endotoxin in parenterals.

Due to the perceived limitations of the previous validation studies, pyrogency testing of biologics and devices and testing for non-endotoxin pyrogens still requires animal-based testing. The two other primary methods for pyrogen testing are the Rabbit Pyrogen Test (RPT) and the Limulus Amebocyte Lysate (LAL). Although the LAL is an *in vitro* test, it is not a non-animal test, as it relies on the blood of horseshoe crabs. The LAL is only capable of detecting endotoxin, which means the RPT is used when non-endotoxin pyrogens are a concern. The LAL has other technical limitations, such as incompatibility with certain types of drugs and biologics that may also lead to the use of rabbits for detection of pyrogenic contamination.

While we appreciate BioTest's intent to expand the use of the MAT in order to replace the RPT, we are concerned about the rabbit use proposed for the validation study. BioTest has suggested a validation study that includes the RPT and LAL along with the MAT. Inclusion of these assays in parallel is an attempt to address the ICCVAM recommendations for future studies enumerated in the 2008 Test Method Evaluation Report (TMER), section 2.3. BioTest also proposes to include endotoxin and non-endotoxin standards (lipotechoic acid and crude preparations from Gram-positive bacteria), a pro-inflammatory substance, parenteral pharmaceuticals, biologics, and devices. However, we question the need for parallel LAL and RPT testing given the inability of the LAL to detect non-endotoxin pyrogens and the abundance of existing LAL and RPT reference data available for comparison and extrapolation.

Product Specific Validation Versus a Large-scale Validation Study

If all the reference standards and classes of products proposed are tested in rabbits, this study could lead to significant animal use. The number of animals who would be consumed by parallel testing is one of the reasons that RPT studies were not conducted as part of the original validation study performed by the European Center for the Validation of Alternative Methods (ECVAM). Another reason cited by ECVAM is the fact that it is common practice for a manufacturer to validate pyrogen tests for every given product. Rather than conducting a massive and animal-intensive validation study, ECVAM opted for a smaller study to demonstrate the general applicability and validity of the methods for regulatory purposes, leaving validation of the assays for additional pyrogens and product classes up to manufacturers. This sensible approach should be applied here to prevent the duplicative use of rabbits in an ICCVAM validation, which would then be followed by a product specific validation anyway. ICCVAM and BioSentinel should take advantage of RPTs currently taking place for regulatory purposes and facilitate product

specific validation of the MAT. Collection of this data could, over time, fulfill data needs for validation of the MAT.

ICCVAM's 2008 Background Review Document (BRD) also acknowledges that product-specific validation of these methods is ultimately part of regulatory practice in the U.S. and in the European Union. Again, this leads us to question whether a large scale validation study including non-endotoxin pyrogens and products beyond parenteral drugs is necessary, particularly since there are numerous studies demonstrating proof of concept for expanded use. There is little doubt that the MAT method nominated, as well as the other 4 versions of the MAT, will work for all pyrogens in a wide array of products (this point is further elaborated later in these comments).

We urge ICCVAM to consider facilitating the expanded use of the MATs via coordination of smaller product-specific validation studies rather than causing additional harm to many animals. ICCVAM could collaborate with FDA to encourage parenteral, biologics, and device manufacturers that still use the RPT to conduct and share the results of their own MAT validation studies. A coordinated effort could build a body of data that would supplant the perceived need for a large prospective study. For example, in 2009, Pfizer and the Center for Biologics Evaluation and Research at FDA published a paper examining the use of certain cell lines for the detection of non-endotoxin pyrogens in a biological product¹. This paper illustrates the type of collaboration and data-sharing that would be useful to advance pyrogenicity testing. One step in this direction is NICEATM's request for data on non-endotoxin pyrogens that appeared in the May 23 issue of the Federal Register. Perhaps this can be used as a starting point to establish collaborations with companies that submit data.

Enhanced Information Gathering

When determining how to proceed, ICCVAM must gather additional information to inform the approach. For example, it would be very useful to know when and how often the RPT is still performed and whether it is used because the LAL will not suffice due to technical limitations or because of a need to address non-endotoxin contamination. If the need to detect non-endotoxin pyrogen is rarely the reason that the RPT is performed, then the need for a validation study of this magnitude is unclear. Other information useful for informing this process includes an understanding of the following: which of the five MATs is most used; whether there is reluctance to use the whole blood-based assays; what product-specific validation studies have been conducted and submitted to FDA.

Scientific Support for the Expanded Use of MATs

There is already very compelling scientific evidence to support the expanded use of MATs without further testing. We question how much more evidence is necessary for ICCVAM to consider the science valid. At the time of the 2008 validation of the MATs for detection of endotoxin in parenteral drugs, there were data available from numerous studies assessing the ability of the MAT's to detect both endotoxin and non-endotoxin pyrogens in a variety of parenterals and some biologics. There were also data available

¹ Huang L, et al. 2009. Use of Toll-Like Receptor Assays to Detect and Identify Microbial Contaminants in Biological Products. *Journal of Clinical Microbiology*. 47(11):3427-3434

directly comparing the MAT with parallel RPTs involving data from hundreds of rabbits. Beyond this evidence, the biological and mechanistic basis of the MATs is well understood. There is little question that the MAT is capable of detecting both endotoxin and non-endotoxin pyrogens^{2,3}.

The basis of the MATs is the detection of particular cytokines, either IL-1 β or IL-6. These cytokines are known to mediate fever in humans. In fact, they are an integral part of the molecular pathways leading to fever, regardless of the source of pyrogen. Over the last 10 years or so, tremendous progress has been made in understanding how the immune system recognizes and responds to pathogens and pyrogens, which are really just fragments of pathogens^{4,5}. In the parlance of modern immunology, pyrogens are referred to as pathogen-associated molecular patterns, or PAMPs. Pyrogens/PAMPs are recognized and bound by a few different Toll-like receptors (TLRs), which sit at the top of convergent molecular pathways leading to fever. All of these pathways involve IL-1 β and IL-6 production as part of the signaling necessary to induce inflammation and fever to deal with microbial threats (Table 1 and Figure 1). Because this anti-microbial response is critical for survival, we have evolved a limited number of conserved signal transduction pathways to mediate this response. By measuring the induction of IL-1 β and IL-6, we can be certain that we are detecting all pyrogenic contamination.

ICCVAM seems to be fixated on the distinction between endotoxin and non-endotoxin pyrogens, but this is an arbitrary distinction in the case of the MATs. These assays are capable of detecting all pyrogens through TLR signaling cascades, leading to induction of IL-1 β and IL-6. The limitation of the LAL, which can only detect endotoxin, may be responsible for the apprehension that is preventing broader application of the MATs. ICCVAM's concerns are misplaced, since MATs are based on a completely different, highly conserved, universal mammalian mechanism--unlike the LAL.

It is illogical to make a dramatic distinction between endotoxin and non-endotoxin pyrogens, while making no such distinction between all the different types of non-endotoxin pyrogens. Endotoxin is one molecular entity, lipopolysaccharide (LPS) from the cell wall of Gram-negative bacteria, but non-endotoxin pyrogens are not one type of substance. Non-endotoxin pyrogens include every other pyrogen aside from LPS, including surface proteins, secreted proteins/toxins, lipoproteins, glycoprotein, lipotechoic acid, peptidoglycan, and nucleic acids. These PAMPs/non-endotoxin pyrogens come from fungal, viral, parasitic, and bacterial (both Gram-positive and Gram-negative) sources. MATs measuring IL-1 β or IL-6 have been shown to detect PAMPs/pyrogens regardless of their composition or origin, which addresses the concern

² Schindler S, et al. 2009. Development, Validation, and Applications of the Monocyte Activation Test for Pyrogens Based on Human Whole Blood. *ALTEX*. 26(4):265-277.

³ Banerjee, S and Mohanan, P.V. 2011. Inflammatory Response to Pyrogens Determined by a Novel ELISA Method Using Human Whole Blood. *Journal of Immunological Methods*. *In Press*.

⁴ Abdul-Sater, A.A. et al. 2009. Inflammasomes Bridge Signaling Between Pathogen Identification and the Immune Response. *Drugs Today*. 45(Suppl. B): 105-112.

⁵ Mogensen, T.H. 2009. Pathogen Recognition and Inflammatory Signaling in Innate Immune Defenses. *Clinical Microbiology Reviews*. 22(2):240-273.

about differentiating between endotoxin and non-endotoxin pyrogens, as well as between the different types of non-endotoxin pyrogens.

Although there are likely to be differences in the timing and levels of cytokine induction between different pyrogens (even from different sources of LPS), these differences can be accounted for by comparing the cytokine profiles arising from LPS and a variety of other PAMPs (i.e. non-endotoxin pyrogens) to ensure the timing of the assay is optimized to detect peak cytokine induction from a broad range of pyrogens. Once optimization is ensured, it is simply a matter of comparing the level of IL-1 β or IL-6 induced by a test substance to the levels induced by a pyrogen standard over a concentration range spanning the fever threshold. Stated another way, the concentration of LPS known to cause fever in rabbits (or humans) can be tested in the MAT and the levels of cytokines produced in response can be used to establish the threshold for gauging pyrogenicity. Bridging between studies in this manner should be sufficient to replace the parallel rabbit testing suggested.

Useful information on the timing of IL-1 β and/or IL-6 induction arising from non-endotoxin pyrogenic contamination may already be available. IL-1 β and/or IL-6 has been well documented using MATs for numerous gram positive species (*Alcalylobacillus acidocaldarius*, *Staphylococcus aureus*, *Corynebacterium diphtheriae*, *Clostridium tetani*, *Bacillus subtilis*, *Bacillus stearothermophilus*, *Micrococcus luteus*, *Lactobacillus plantarum*, group B streptococcus, and *Streptococcus pneumoniae*), mycobacteria, and numerous fungal species (*Candida albicans*, *Aspergillus niger*, *Aspergillus versicolor*, *Alternaria alternate*, *Cladosporium cladosporoides*, and *Penicillium crustosum*). Induction of IL-1 β and/or IL-6 have also been demonstrated for specific non-endotoxin pyrogens/ PAMPs including lipoteichoic acid, diphtheria toxin, tetanus toxin, spores of Gram positives and fungi, as well as peptidoglycan and other constituents of both Gram-positive and Gram-negative cell walls. Furthermore, many of these analyses were performed using biologics and devices as well as parenterals, so there is a strong precedent for the detection of non-endotoxin in products other than parenteral drugs.

Need for an Updated Literature Review

Before proceeding with further validation of the nominated MAT, we strongly recommend conducting an updated review of the literature. We are surprised and disappointed that a more exhaustive review of current findings was not submitted in support of the nomination. We hope that the 2008 BRD and TMER are not relied upon for information on the state of the science. The TMER contains outdated references and reaches some misinformed conclusions, for example "The development of tests based on the production of such cytokines [IL-1 β , IL-6, and TNF] from human white blood cells or cell lines appears to correlate well with the induction of fever in both the RPT and humans. However, the RPT detects a whole organ/body fever response; whereas, the proposed test methods detect only cytokine secretion. Evidence to suggest that detection of IL-1 β or IL-6 is necessarily an indication of a febrile reaction is lacking."⁶ Numerous

⁶ Hoffman, H.M. and Brydges, S.D. 2011. Genetic and Molecular Basis of Inflammasome-mediated Disease. *Journal of Biological Chemistry*. 286(13):10889-10896.

studies and reviews have been published in the last several years to address these concerns and significantly inform the validation process.

We hope that, in accordance with ICCVAM's Congressional mandate, the increased use of MATs can be achieved quickly and simply. It is troubling that approval for an expanded domain of applicability is still an issue, given the extensive scientific evidence to support detection of non-endotoxin pyrogens in varied products. The MATs are affordable and practical, based on straightforward, well-established, and simple ELISA "technology" that is widely used throughout even the most modestly equipped labs. There appear to be few downsides to the MATs, which detect a broader array of pyrogens than the LAL and are more sensitive than the RPT.

Botulinum Neurotoxin (BoNT) Activity Assays

Clostridium botulinum toxin testing applications range from food safety needs to vaccine potency-type tests, and also include field tests for outbreaks in lakes and in animals such as waterfowl, horses, cows, domestic poultry, fish, and fish-eating birds. Botulinum toxin is produced by *Clostridium botulinum*, a gram positive, spore-producing, anaerobic bacterium that is capable of producing up to seven different serotypes of botulinum toxin. It is a potent neurotoxin that: (1) can contaminate food sources, (2) can be used in pharmaceutical applications, and (3) is also thought to be a concern related to military defense applications. Types A, B, E, and F are inherited chromosomally, while types C and D are transmitted to *C. botulinum* via bacteriophages, and type G is exchanged on plasmids. Serotype A is used most often in pharmaceutical preparations.

United States regulatory and governmental agencies such as the Food & Drug Agency's (FDA) Center for Food Safety and Applied Nutrition (CFSAN), FDA's Center for Biologics Evaluation & Research (CBER) and The US Geological Survey's National Wildlife Health Center (USGS NWHC) currently rely on the mouse bioassay for detecting *C. botulinum*-based toxins. It has long been proposed that a user-friendly, rigorous, non-animal-based replacement to the mouse bioassay (MBA) is needed due to ethical concerns as described below.

The MBA for *C. botulinum* toxin detection requires a dilution series of the toxin to be injected intraperitoneally into multiple mice. The resulting poisoning causes an often slow and painful death, ultimately culminating in respiratory paralysis. The MBA requires at least 48 mice for testing in food safety and approximately 100 mice per potency test for a single batch of Botox. The MBA accounts for the use of an estimated 74,000 mice in a single Botox manufacture's lab in one year and for 600,000 mice used worldwide per year by pharmaceutical companies, alone.

In addition to animal welfare concerns, the MBA also has scientific and practical shortcomings. One study in California found that only 68% of tests run on the serum from 73 patients with wound botulism resulted in positive MBA tests⁷, illustrating a

⁷ Wheeler C, Inami G, Mohle-Boetani J, Vugia D. Sensitivity of mouse bioassay in clinical wound botulism. Clin Infect Dis. 2009;48:1669-73.

significant false negative rate by the MBA. The MBA is incapable of distinguishing BoNT serotype unless neutralization tests with each antisera are carried out in parallel. Additionally, the MBA takes a week to complete, requires trained staff, ample cage space within animal facilities, licensing for the use of the mice, and considerable costs for the week-long test and the staff to carry it out.

In order to move past reliance on the MBA, a desirable test method candidate for monitoring and quantifying *C. botulinum* toxins must be: (1) indicative of active toxin(s), (2) inexpensive, (3) rapid, and (4) sensitive, and have the capability to be used in field monitoring. ICCVAM received a nomination to evaluate three botulinum neurotoxin detection methods developed by BioSentinel, Inc., (Madison, WI) and has requested public comment on each of the three methods.

BoTest™

BoTest™ botulinum neurotoxin (BoNT) detection kits are capable of detecting *C. botulinum* A, B, D, E, F and G proteolytic activity. BoTest™ is intended to be used with purified samples and for drug discovery. Depending on the serotype being tested, BoTest™ offers mouse-level or near mouse-level sensitivity. In contrast to the MBA, the detection capabilities by BoTest™ are in real time, the output signal wavelength is tunable, and the system requires only a small amount of training to use.

A pharmaceutical company has validated BoTest™ assays for the quantification of drug formulations and products and is in the process of performing comparability studies between BoTest™ and the MBA. This valuable data should be used by ICCVAM as part of the requirement for method validation.

Currently, specific BoTest™ substrates are available for six of the seven serotypes of botulinum toxins. BoTest™ A/E uses a SNAP-25-based reporter, while BoTest™ B/D/F/G assay uses synaptobrevin specific for those subtypes. The BoTest substrates are comprised of much larger fragments from the substrate proteins than competing commercial assays and therefore the BoNT has much higher affinity for these substrates than those used in other commercially prepared reporter systems, although the data to support this was not provided in the supporting documents. Because these assays quantify the endopeptidase activity of the BoNT using reporters linked to modified endogenous BoNT targets, the BoTest™ assays are biologically relevant.

BoTest™ takes advantage of Forster Resonance Energy Transfer (FRET) donor-acceptor pair fluorescence. BoNT cleavage of the substrate leads to decrease of visible yellow fluorescent protein (YFP) emission with a concomitant increase in cyan fluorescent protein (CFP) emission. The reactions are measurable in real-time and emissions can be quantified and enzymatic activity can therefore be determined.

Cost comparison by BioSentinel of BoTest™ with the MBA shows BoTest™ is much less expensive, at a cost \$875.00 per test (including labor) compared with an estimated \$6000.00 for the MBA, which would also result in the suffering and death of up to 300

mice. BoTest™ can be completed in as little as two hours while the mouse test commonly takes a week or more.

BoTest™ is biologically relevant and has sensitivity in the femtomolar and picomolar range, making this method unique among commercially available assays and also a strong contender to completely replace animal testing as part of a suite of MBA replacement assays.

BoTest™ Matrix

BoTest™ Matrix kits were specifically designed to be used in complex matrices (blood, serum, water, pharmaceutical products, and food). The Matrix test kits can detect botulinum A and E serotypes and use magnetic beads to capture and concentrate BoNT-containing matrices.

The reporter substrates are the same as those used for BoTest™ and also use FRET detection of reporter cleavage in real time. Sensitivity rivals that of the MBA (femtomolar and picomolar range), but is higher throughput. Costs for the BoTest Matrix™ kit is estimated \$1930.00 at a commercial laboratory while the MBA would cost \$6000.00. The BoTest Matrix™ kits are readily transferable between labs and require little training and, like the BoTest™, take as little as two hours to complete compared to a week for the MBA.

BoCell™

The BoCell™ A assay uses an engineered cell line that responds to intracellular BoNT proteolytic activity by use of a stably transfected reporter and is specific for serotype A. The BoCell™ assay is intended to be used as a direct replacement for the mouse bioassay. The substrate is a SNAP-25-based sequence fused to two fluorescent reporter proteins. BoNT cleavage activity is detected by either a loss FRET pairing or by destruction of the C-terminal fluorophore.

BoCell™ can be carried out in any lab capable of basic tissue culture and requires minimal training, which is in contrast to the training and certification required for the MBA.

BoCell™ offers a high throughput method of BoNT detection without use of any animals and with minimal equipment. At this time, the BoCell™ is not as sensitive as the MBA (2 – 3 orders of magnitude less sensitive), but for many applications, this level of sensitivity is acceptable. BioSentinal is recommending BoCell™ in combination with BoTest™ or BoTest Matrix™ assays for applications that require increased sensitivity. Depending on the application, the combination of two or three of the assays can meet the needed specificity and sensitivity.

Cost estimates are not completed, but running costs are thought to be in line with maintaining a typical adherent cell line. BoCell™ can be completed in 24 to 96 hours, depending on the application while the MBA typically takes a week to complete.

As a complete suite of BoNT-detecting and quantifying assays, the BoTest™, BoTest Matrix™, and BoCell™ assays appear to be quite promising and deserving of ICCVAM-sponsored validation. These assays are capable of saving hundreds of thousands of mice from painful deaths while protecting food supplies, monitoring susceptible wildlife, and quantifying Botox batch potency.

ICCVAM must follow its Congressional mandate and implement the validation of this cost and life-saving suite of assays with all due speed, thus eliminating the use of mice for *C. botulinum* toxin detection and quantification. In planning this validation study, we request that ICCVAM use the available data showing BoTest™'s capabilities with respect to quantifying drug formulations and related products, as well as any data that the company has gathered regarding comparability between BoTest™ to the MBA, and apply these data sets towards validation efforts.

Thank you for your attention to these comments on validation of Botulinum neurotoxin activity assays and expanded validation of a MAT pyrogencity assay. We can be reached for questions at the contact information below.

Sincerely,

[Redacted]

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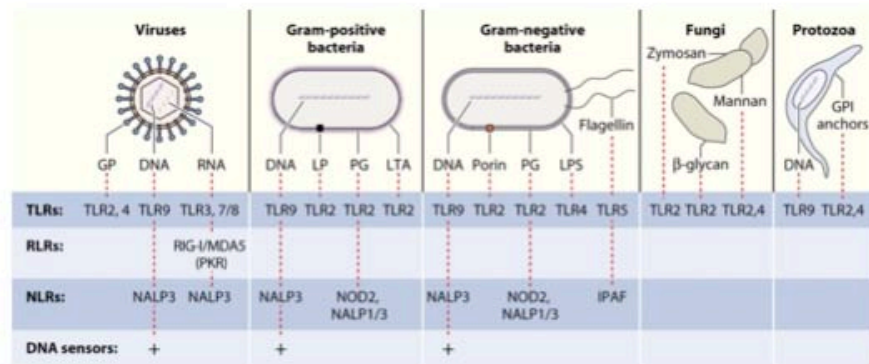
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Table 1: Recognition of Pyrogens by TLRs

Receptor	Cellular localization	Microbial component(s)	Origin(s)
TLRs			
TLR1/TLR2	Cell surface	Triacyl lipopeptides	Bacteria
TLR2/TLR6	Cell surface	Diacyl lipopeptides	<i>Mycoplasma</i>
		Lipoteichoic acid	Gram-positive bacteria
TLR2	Cell surface	Lipoproteins	Various pathogens
		Peptidoglycan	Gram-positive and -negative bacteria
		Lipoarabinomannan	Mycobacteria
		Porins	<i>Neisseria</i>
		Envelope glycoproteins	Viruses (e.g., measles virus, HSV, cytomegalovirus)
		GPI-mucin	Protozoa
		Phospholipomannan	<i>Candida</i>
		Zymosan	Fungi
		β -Glycan	Fungi
TLR3	Cell surface/endosomes	dsRNA	Viruses
TLR4	Cell surface	LPS	Gram-negative bacteria
		Envelope glycoproteins	Viruses (e.g., RSV)
		Glycoinositolphospholipids	Protozoa
		Mannan	<i>Candida</i>
		HSP70	Host
TLR5	Cell surface	Flagellin	Flagellated bacteria
TLR7/8	Endosome	ssRNA	RNA viruses
TLR9	Endosome	CpG DNA	Viruses, bacteria, protozoa

Mogensen, T. H.. 2009. Clin. Microbiol. Rev. 22(2):240-273

FIG. 1. Recognition of PAMPs from different classes of microbial pathogens



Mogensen, T. H.. 2009. Clin. Microbiol. Rev. 22(2):240-273